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Carbohydrate Starvation Causes a Metabolically Active but Nonculturable State in *Lactococcus lactis*▽†‡

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This study characterized the ability of lactococci to become nonculturable under carbohydrate starvation while maintaining metabolic activity. We determined the changes in physiological parameters and extracellular substrate levels of multiple lactococcal strains under a number of environmental conditions along with whole-genome expression profiles. Three distinct phases were observed, logarithmic growth, sugar exhaustion, and nonculturability. Shortly after carbohydrate starvation, each lactococcal strain lost the ability to form colonies on solid media but maintained an intact cell membrane and metabolic activity for over 3.5 years. ML3, a strain that metabolized lactose rapidly, reached nonculturability within 1 week. Strains that metabolized lactose slowly (SK11) or not at all (IL1403) required 1 to 3 months to become nonculturable. In all cases, the cells contained at least 100 pM of intracellular ATP after 6 months of starvation and remained at that level for the remainder of the study. Aminopeptidase and lipase/esterase activities decreased below detection limits during the nonculturable phase. During sugar exhaustion and entry into nonculturability, serine and methionine were produced, while glutamine and arginine were depleted from the medium. The cells retained the ability to transport amino acids via proton motive force and peptides via ATP-driven translocation. The addition of branched-chain amino acids to the culture medium resulted in increased intracellular ATP levels and new metabolic products, indicating that branched-chain amino acid catabolism resulted in energy and metabolic products to support survival during starvation. Gene expression analysis showed that the genes responsible for sugar metabolism were repressed as the cells entered nonculturability. The genes responsible for cell division were repressed, while autolysis and cell wall metabolism genes were induced neither at starvation nor during nonculturability. Taken together, these observations verify that carbohydrate-starved lactococci attain a nonculturable state wherein sugar metabolism, cell division, and autolysis are repressed, allowing the cells to maintain transcription, metabolic activity, and energy production during a state that produces new metabolites not associated with logarithmic growth.

Carbohydrates are the primary energy and carbon sources for lactic acid bacteria (LAB) during growth in laboratory media and fermented products, such as milk. These traits are associated with specific plasmids that have distinct genes for proteolysis and lactose metabolism (35). During the fermentation processes, LAB are subject to the vagaries of stress due to changes in water activity, pH, redox potential, and substrate availability (38). Lactococci respond to stress conditions by regulating many metabolic pathways to maintain metabolism and energy production (61). In response to carbohydrate starvation, lactococci become nonculturable (NC) and remain metabolically active for at least 2 weeks (48).

At the onset of carbohydrate starvation in lactococci, the intracellular levels of the glycolytic intermediates, phosphoenolpyruvate (PEP), 3-phosphoglycerate, and 2-phosphoglycerate increase, all of which constitute the PEP potential (53, 54). During persistent starvation, PEP is metabolized to pyruvate and ATP (53, 54). Thompson and Thomas (54) suggested that lactococci utilize intracellular glycolytic reserves for moderate periods of starvation to compensate for the lack of sugar. The PEP potential allows enzymatic activity and transport to remain active for survival during the depletion of carbohydrates and is used to reactivate sugar metabolism.

The ability to form colonies on solid media is lost at the onset of carbohydrate starvation (48). During this NC state, cells are incapable of sustaining cellular division required for growth on rich nonselective media (39) but do remain metabolically active for extended times. Many genera exhibit the NC state, including *Escherichia coli*, *Micrococcus luteus*, *Vibrio vulnificus*, *Campylobacter jejuni*, and *Brevibacterium linens* (3, 39). However, the duration of survival in the NC state has not been demonstrated past 30 days in lactococci, thereby questioning the role of cells in this cellular state in long-term survival during stress and hence the role of this cellular stage in the flavor development of fermented foods during storage. While the genes involved in abiotic stresses, such as pH and temperature, are well characterized in lactococci (42–44, 61), the molecular events associated with the NC state are not delineated.

Macromolecular metabolism decreases in NC bacteria, similar to the response of marine bacteria to starvation (29). However, starved cultures are not metabolically static cells. Changes during or after transition to the NC state include changes in the size and shape of the bacteria to minimize

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energy requirements, a decrease in the number of ribosomes, and changes in fatty acid content of the cell membrane in response to local conditions (39, 58). The NC cells of lactococci can synthesize RNA (51). Thus, the cells appear to decrease in number, or even die, by classical plating techniques, but they remain metabolically active (48).

Lactococci are capable of using alternate carbon sources such as RNA, lipids, proteins, peptides, and amino acids for energy during carbohydrate starvation (49). Carbohydrate starvation and energy depletion prompt lactococci to shift their metabolism from glycolysis to amino acid catabolism (48). The genes forming the arginine deiminase pathway are present on the chromosome in lactococci (8, 9) and serves as either the sole or an additional source of energy, carbon, and nitrogen in LAB, *Bacillus*, *Pseudomonas*, *Aeromonas*, *Mycoplasma*, clostridia, and halobacteria (13, 14). This pathway produces 1 mol of ATP per mol of arginine in carbohydrate-depleted medium (13, 14). This additional source of ATP increases the survival of *Lactococcus lactis* (48).

During sugar starvation, the available energy within cells is utilized for protein and biomolecule synthesis rather than for the generation of cell mass. These proteins are then degraded by lactococci over time to generate peptides and amino acids that aid survival (50, 51). Aminopeptidases (APs) aid protein turnover and new protein synthesis at transitional states during starvation (26). Hence, protein metabolism and AP activity may be limiting factors for survival.

The ability of lactococci to survive during carbohydrate starvation and continue transcriptional and translational turnover also indicates their ability to actively metabolize proteins and amino acids (51). They shift away from lactic acid production toward nitrogenous metabolism in the NC state, which leads to new metabolic end products, such as fatty acids (21–23). These changes manifest as a phenotype of persistent nonculturability.

In this study, we hypothesize that carbohydrate starvation represses specific genes for replication without induction of the lytic genes in lactococci. To test this hypothesis, this study compared orthogonal cellular responses in three lactococcal strains during carbohydrate starvation by using plate counts, intracellular ATP content, a fluorescent probe for membrane compromise, amino acid metabolism, enzyme activity, macromolecule transport, and gene expression profiles to determine multiple metabolic responses to starvation. The findings indicate that lactococci remained metabolically active by maintaining membrane integrity, shifting the metabolism from carbohydrates to amino acids, and maintaining ATP levels during the NC state. The population remained capable of amino acid catabolism and yet was NC on solid agar for at least 3 years. Gene expression profiles measured across the cellular phases (logarithmic phase, starvation, and nonculturability) showed that, among many expression changes, the onset of starvation repressed genes for sugar catabolism and cell division. Genes associated with cell lysis (those encoding *N*-acetyl muramidases, lysins, and holins) were not expressed at any phase of cell growth.

MATERIALS AND METHODS

Bacterial strains and media. *Lactococcus lactis* subsp. *lactis* ML3 and IL1403 and *Lactococcus lactis* subsp. *cremoris* SK11 were obtained from the Utah State University culture collection. The *L. lactis* subsp. *lactis* strains were propagated in Elliker's and M17G broths, respectively, and *L. lactis* subsp. *cremoris* SK11 was grown in M17L broth (Difco Laboratories, Detroit, MI). Stock cultures were prepared by growing the organisms twice in 10 ml of the respective broth at 30°C for 24 h. Cultures were frozen for storage at −70°C in 10% nonfat dry milk containing 30% glycerol. Before each experiment, frozen stock cultures were thawed and subcultured twice at 30°C for 24 h in 10 ml of the respective broth.

The strains were grown overnight at 30°C, harvested by centrifugation (6,000 × *g* for 15 min at 4°C), washed twice with and resuspended in sterile saline, and inoculated (1%) into sterile chemically defined basal medium (CDM) (24). For short-term starvation studies, the basal CDM was supplemented with 0.1% lactose. For long-term starvation, the basal CDM contained 0.2% lactose or glucose, and branched-chain amino acids (BCAAs) were added at 10 times the original CDM content of 20 mg/liter. The CDM was adjusted to either pH 7.2 buffered with 0.19 M of sterile 3-(*N*-morpholino)propanesulfonic acid (MOPS) or pH 5.2 buffered with 0.19 M 2-(*N*-morpholino)ethanesulfonic acid and filter sterilized. Amino acids and buffer salts were purchased from Sigma (Sigma-Aldrich, St. Louis, MO).

Lactose and glucose determination. Lactose or glucose concentration was determined either by the colorimetric method of Dubois et al. (16) or by high-pressure liquid chromatography as described by Stuart et al. (48).

Culturable cell estimation. Samples were taken at determined time points from the culture suspensions and diluted in sterile saline dilution blanks. These dilutions were either spiral plated as described by Stuart et al. (48) or plated on the respective solid medium by using the spread-plate technique in duplicate. The plates were incubated anaerobically for 48 h at 30°C. Colony counts were determined in spiral-plated plates according to the manufacturer's instructions by using duplicate plates.

Cell viability estimation by fluorescence. Samples were collected from the cell suspension, and bacterial viability was estimated as described previously by Stuart et al. (48).

ATP determination. Intracellular ATP concentration in cell suspensions was quantified by measuring bioluminescence with an ATP assay kit as described in the manufacturer's instructions (Calbiochem-Novabiochem Corporation, San Diego, CA) and was described earlier (48). Luminescence was measured on an LS6500 scintillation counter (Beckman Coulter Inc., Fullerton, CA).

AP and lipase/esterase (LE) activities. Cell-free extracts (CFE) from cultures were prepared as described by Dias and Weimer (15) using sterile 106-μm glass beads (Sigma Chemical Co., St. Louis, MO) to facilitate cell lysis. The samples were vortexed at high speed for 10 min at 1-min intervals, with alternate dipping in an ice bath. The supernatant was collected and used as the CFE for intracellular enzyme assays. The protein content of the CFE was determined spectrophotometrically using a bicinchoninic acid assay according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL). Bovine serum albumin was used to obtain a standard curve.

AP activity was measured by a semiautomated colorimetric method using reflectance colorimetry as described by Dias and Weimer (15). The assay mixtures contained 100 μl of 1 mM chromogenic substrate in 0.05 M sodium phosphate buffer (pH 7.2) and 100 μl of CFE. Hydrolysis of the chromogenic substrate was measured as the increase in yellowness (b^*) by using a reflectance colorimeter (Omnispec 4000 bioactivity monitor; Wescor, Inc., Logan, UT) every 5 min for 3 h at 30°C in duplicate.

LE activity was determined by a semiautomated reflectance colorimetric method using *p*-nitrophenyl derivatives (Sigma-Aldrich, St. Louis, MO) of butyrate and caprylate (1). Each assay mixture contained 20 μl of 1 mM chromogenic substrate in 80 μl of 0.05 M sodium phosphate buffer (pH 7.2) with 0.2 mM Triton X-100 and 100 μl of CFE. Colorimetric measurements were determined as described for AP activity.

Enzyme activities were determined by plotting the change in yellowness (Δb^*) over the assay time (15). The linear portion of the curve was used to calculate the slope. The slope was divided by the amount of protein in the added CFE to give the AP and LE activities (Δb^*/mg protein/h).

Amino acid determination. CDM samples were prepared and derivatized with 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde as described by Stuart et al. and Ummadi and Weimer (48, 55). Norleucine was added as an internal standard to each reaction mixture before the derivatizing agent was added. Amino acids were monitored by micellar electrokinetic chromatography and laser-induced fluorescence (48, 55). The results are presented as amino acid concentrations (mM).

Peptide metabolism. After 8 months of starvation, a peptide uptake and utilization assay was performed using the casein peptide $\alpha s_{1\text{-}9}$. Cells were collected from CDM by centrifugation on a bench-top centrifuge at 12,000 × *g* for 2 min and washed once with 190 mM MOPS at pH 7.2. The cells were resuspended in the same buffer containing the peptide, and the level of peptides in the

assay buffer was monitored at 0, 1, 3, and 24 h by high-pressure liquid chromatography as described by Broadbent et al. (4).

Amino acid metabolism assay. The cells were collected at one specific time point and incubated with [2-^{13}C]leucine as described by Ganesan et al. (22, 23). Postincubation, the supernatant and cell pellet were collected by centrifugation (12,000 × *g* for 2 min) and the products were assayed in the supernatant and cell extracts by nuclear magnetic resonance spectroscopy as described by Ganesan et al. (23).

Gene expression analysis. A whole-genome array was designed, produced, and validated by NimbleGen Systems, Inc. (Madison, WI) using the genome sequence of IL1403 that is publicly available (2). RNA samples at logarithmic phase, the onset of starvation, and nonculturability were extracted, purified, and reverse transcribed to cDNA as described by Xie et al. (61). The cDNA was sheared enzymatically by the protocol of Nimblegen Systems, Inc. and biotinylated as described by Xie et al. (61) prior to hybridization. The cDNA samples were hybridized and detected at NimbleGen using the NimbleScreen oligonucleotide microarrays designed and optimized for IL1403.

Statistical analysis and gene expression visualization. All experiments were done in two biological replicates. Starvation physiological and metabolic data from lactococcal strains ML3, SK11, and IL-1403 were analyzed with time as a repeated measure to determine strain-wise treatment effects (equation 1). The factors used in statistical analyses were the effect of time, the effect of pH, their combined effect on strains, and the differences between any two strains at different pHs. SAS statistical software, version SAS 9.0 (SAS Statistical Institute, Cary, NC), was used for the analysis. The parameters used in statistical analyses were plate count estimates, live and dead cell counts, ATP content, and AP/LE activities. Significant differences were assigned an α value of 0.05. All the *P* values obtained were multiplied by the total number of time points (30) to correct for multiple comparisons.

$$Y_{ijk} = \text{mean} + \text{pH}_i + \text{time}_j + (\text{pH} \times \text{time})_{ij} + (\text{error})_{ijk} \quad (1)$$

where *Y* is the response variable generalized in the previous sentence and *i*, *j*, and *k* are the parameters in the equation.

For gene expression data, the raw pixel intensities from all arrays were simultaneously normalized using the Robust Multichip Average package within R statistical software, version 2.1.0. The $\log_2$ values of Robust Multichip Average-normalized data were averaged across replicates and plotted as expression maps in Hierarchical Clustering Explorer software, version 3.0 (46), for visualization. Coloring of the expression maps was based on whole-genome expression levels for all samples. A baseline was determined at the software-calculated mode that was color-coded green. Two levels of expression changes were set 2.5-fold apart from each other and were consequently represented by black and red.

The statistical significance of gene expression changes was determined using R statistical software suite v2.0, using a repeated-measure, linear mixed-effects model with a compound symmetry error structure with the lme package. The false discovery rate to adjust against multiple comparisons was determined using the qvalue package in R software. The false discovery rate (*q* value) was set at 30%.

RESULTS

The phenomenon of nonculturability is difficult to verify since the traditional measure of growth on a solid medium is the standard assay. However, the use of 16S DNA sequencing and metagenomics indicates that the NC state is common in extreme environments (56). Due to the difficulty of determining this bacterial state, this study used numerous lines of evidence relevant to the physiology of lactococci in addition to growth to estimate the NC state. Each method of analysis provides a unique perspective of the metabolic state and cellular capability during growth and carbohydrate starvation. This approach provides evidence about the general health of the cell to prove that the NC state and the cellular capabilities for survival and metabolism exist in lactococci.

Growth and starvation survival. Three lactococcal strains were tested for survival and growth during short-term starvation (42 days) and long-term starvation (3.5 years). These strains were selected based on different rates of lactose metabolism (48). Strain variation was tested using a common laboratory strain (IL1403) that is plasmid free, a commercial strain commonly used in the laboratory (ML3) that has three plasmids (including *lac* and *prt*), and a commercial strain (SK11) that has five plasmids (35). Two pH conditions were tested to simulate laboratory conditions (pH 7.2) and the acidic stress of cheese ripening (pH 5.2), their industrial use. The pH stress response leads to cross-protection for other stresses in lactococci (61). To control for this confounding effect, we incubated the cells under both pH conditions to determine whether other stresses play a role in nonculturability.

Plate count assays were done to determine the culturability of each strain during the study. IL1403 was used as the control organism since it is plasmid free and lacks the ability to metabolize lactose (2) due to the absence of the *lac* plasmid. As such, the addition of lactose to the growth medium of this strain mimics carbohydrate starvation. This was tested by comparing the addition of lactose to glucose catabolism in IL1403 as a control for the role of plasmids. Buffered CDM was used to avoid confounding the effect of pH stress during the study.

Culturable cell estimation and sugar metabolism. During short-term starvation, the cell density of ML3 increased significantly ($P \leq 0.05$) (Fig. 1). *L. lactis* subsp. *lactis* ML3 metabolized the added lactose to below detectable levels within 1 day (Fig. 2A) and did not change the pH of the buffered medium. However, ML3 became NC at 8 days, 7 days after lactose depletion, suggesting that additional molecular events were occurring to allow this strain to survive. Interestingly, the addition of 0.2% lactose to the medium of ML3 again resulted in complete utilization within 1 week (Fig. 2A). This addition produced a reduction in pH of ~0.5 units under both pH conditions tested, which agrees with the observations of Chou (8). SK11 utilized all the available lactose at pH 7.2 but did not utilize any lactose at pH 5.2 (Fig. 2), nor did it alter the pH in either medium. IL1403, being plasmid free and incapable of metabolizing lactose, did not utilize lactose at either pH (Fig. 2A), as expected, but IL1403 utilized the glucose within 1 day at pH 7.2.

ML3 metabolized lactose quickly (i.e., within 1 day) and became NC within 9 days under both pH conditions. Alternatively, SK11 and IL1403 utilized sugar slower than ML3 and were culturable at pH 7.2 until 30 days and 112 days, respectively (Fig. 1B). When grown at pH 5.2, SK11 became NC at 11 days, while IL1403 did not become NC until 240 days (Fig. 1C). IL1403 in glucose at pH 7.2 attained nonculturability at 21 days, which was similar to what was observed for SK11. These data demonstrate that a relationship exists between the metabolism of carbohydrates and the NC state in lactococci, which is strain dependent. These results were expected because carbohydrate metabolism generates ATP and influences cellular growth and density (8, 33). This was also observed by Chou (8), who found that the ratio of sugar to arginine influenced nonculturability and the final pH of the medium due to the production of acid from sugar and that of ammonia from arginine, respectively. These observations further suggest that genetic regulation switches between carbohydrate metabolism and other metabolic modes determine the time at which a cell reaches the NC state. The metabolic switch is likely to be regulated by *ccpA*, which is known to act as both a repressor and an activator between carbohydrate and amino acid metabolisms (25).

Viability. To verify that the cells were viable without culturing, a cellular membrane probe that produces fluorescence upon binding with DNA was used to examine membrane in-

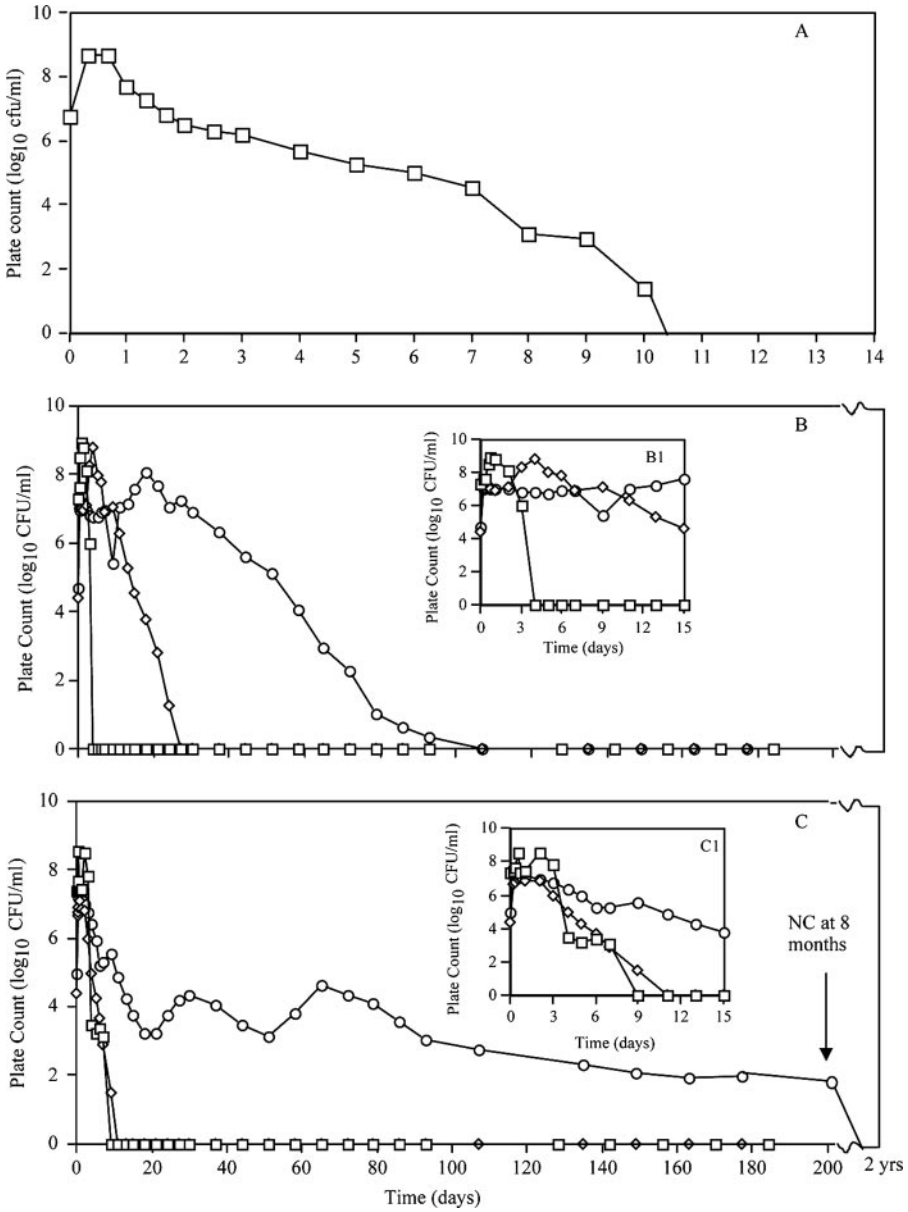

FIG. 1. Cell counts during growth and carbohydrate starvation in buffered CDM. Plate counts of ML3 in buffered CDM containing 0.1% lactose at pH 7.0 (squares) (A) and plate counts of ML3 (squares), SK11 (diamonds), and IL1403 (circles) in CDM at pH 7.2 (B) and within the first 15 days of starvation (B1) are shown. Plate counts of ML3 (squares), SK11 (diamonds), and IL1403 (circles) in CDM at pH 5.2 (C) and within the first 15 days of starvation (C1) are also shown. ML3 data are shown for up to 2 years, but ML3 was NC for up to 3.5 years. The coefficient of variation ranged between 0.1 and 9% for all strains at each time point and under each pH condition.

tegrity as demonstrated by Stuart et al. (48) for lactococci. The relative fluorescence of the live populations for all cultures increased with growth in liquid media. The measure was the same for logarithmic-phase cells and NC cells (see the supplemental material) during short- and long-term starvation studies. After the NC state was attained, the viabilities of all strains remained at similar levels. Further observations for 2 years in CDM showed that the cells remained NC with no change in membrane integrity (see the supplemental material). These observations are similar to those of Stuart et al. (48), except that this study demonstrated that the cells maintained intact membranes in the NC state for at least 2 years. This indicates that lactococci are capable of maintaining the membrane structure for extended periods of starvation and suggests that intracellular processes remain active for cellular metabolism, including the preservation of ATP and a proton motive force (PMF) for transport.

Cellular energy. The ATP concentration during growth was determined to estimate the amount of cellular energy available during logarithmic growth and nonculturability since the cells maintained intact membranes. During short-term starvation, the ATP concentration of ML3 followed a pattern similar to cell count estimations (Fig. 2B) and significantly increased over time ($P \leq 0.05$). The ATP level rose during lactose metabolism

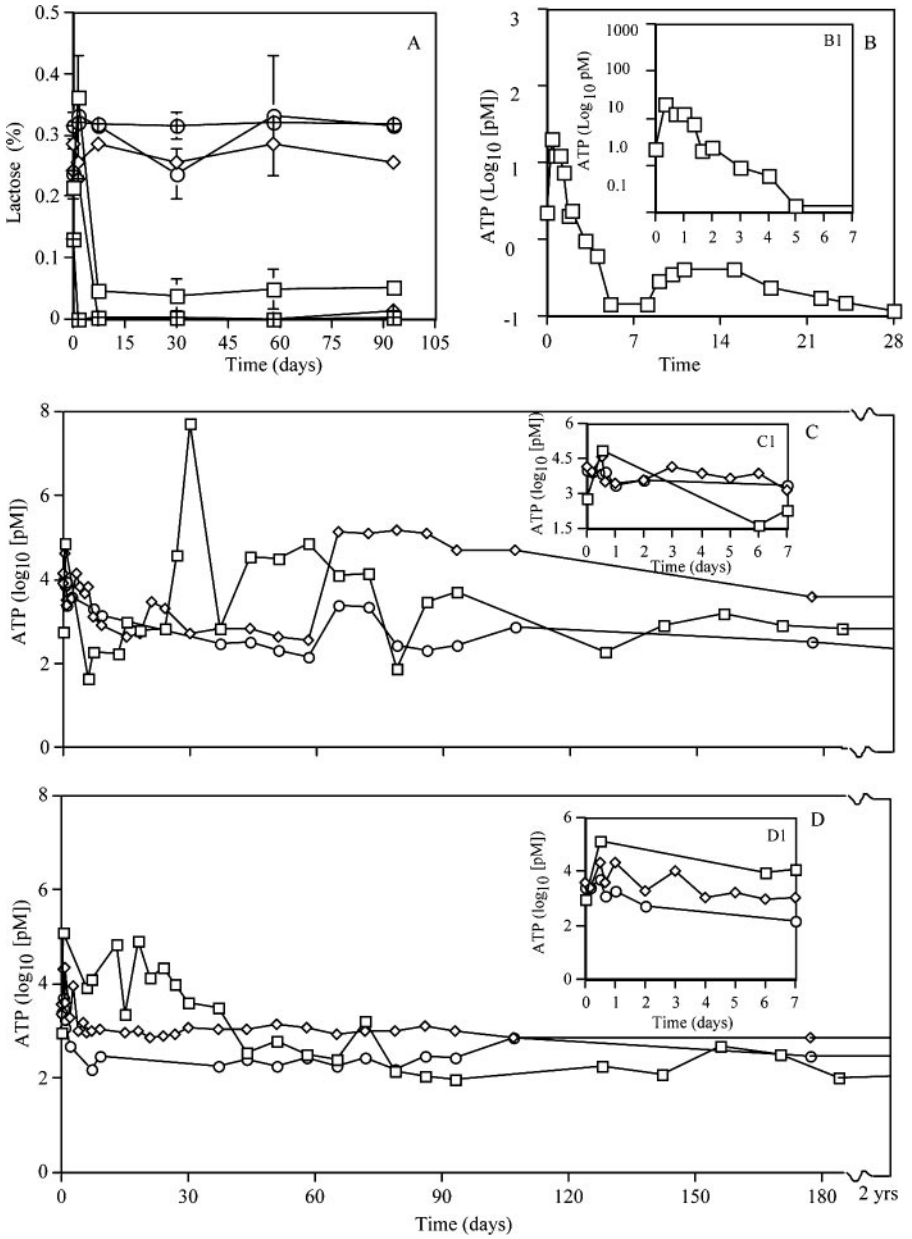

FIG. 2. Lactose utilization during growth and nonculturability in buffered CDM and intracellular ATP concentrations of cells. The initial lactose level was 0.2 to 0.25% for all media. (A) Lactose levels during long-term starvation of ML3 at pH 7.2 (filled squares) and pH 5.2 (open squares), SK11 at pH 7.2 (filled diamonds) and pH 5.2 (open diamonds), and IL1403 at pH 7.2 (filled circles) and pH 5.2 (open circles). (B) Lactose levels of ML3 at pH 7.0 during short-term starvation. Data are shown for 93 days, while lactose levels were maintained for 3.5 years. ATP levels of ML3 (squares), SK11 (diamonds), and IL1403 (circles) in CDM at pH 7.2 (C) and within the first 7 days of starvation (C1) are shown. ATP levels of ML3 (squares), SK11 (diamonds), and IL1403 (circles) in CDM at pH 5.2 (D) and within the first 7 days of starvation (D1) are also shown. ML3 data are shown for up to 2 years, but ML3 contained 100 pM ATP for up to 3.5 years. The coefficient of variation ranged between 0.8 and 11% for all strains at all time points and under all pH conditions tested.

but declined after lactose depletion to a constant level of about 0.1 pM for 42 days (Fig. 2B).

During long-term starvation, the strains produced ATP during the growth phase under both pH conditions (Fig. 2C and D) in ML3 and SK11. Interestingly, during long-term starvation, ATP levels were relatively constant and were never below 100 pM. This likely represents the basal level of ATP that allows the cells to maintain metabolism, even in the absence of colony formation. These data suggest that cells contained adequate ATP for metabolic activities under starvation conditions, even though the source of ATP during logarithmic growth (i.e., lactose) was metabolized after 1 day or within 1 week depending on the strain (Fig. 2A).

Metabolic activities. To verify that the cells were metabolically active and cellular metabolism was active after the extended incubation, a series of intracellular assays were done

that measured various metabolic activities of the cell that required energy. All assays were used to demonstrate the ability of NC cells to remain metabolically active, despite their inability to produce colonies on a solid medium.

Peptide metabolism. The transport mechanism of casein-derived peptides via the Opp system that requires ATP is chromosomal (4, 10). During long-term starvation, peptide uptake with ML3 was determined at 8 months of incubation in an assay system to verify the presence of adequate ATP so as to allow active transport during the NC state. ML3 initiated transport of the $\alpha s_{1\text{-}9}$ casein peptide within 3 h of exposure to the peptide and completely utilized the peptide within 24 h (data not shown). This confirmed that NC cells have membrane integrity (see the supplemental material), contain sufficient ATP (Fig. 2), and have active peptide transport systems (i.e., the summation of Opt, Opp, and Dpp oligopeptide transport systems) that require ATP.

AP and lipase activities. Once the peptide is transported into the cell, AP degrades them into the constituent amino acids. This feature is important to industrial fermentations for growth and flavor production during product storage. During short-term starvation, AP and LE activities were present in ML3 initially at 80 to 90 units Δb^*/mg protein/h (data not shown). However, these activities significantly ($P \leq 0.05$) decreased to undetectable levels by 6 days. The decrease in activity of AP and LE may be due to a decrease in the energy needed for these enzymes to function or for transcription. Alternatively, the lack of triacylglycerols and peptides or increased free amino acids inhibits the activities and expression levels of AP and LE (36), which were present in this defined medium. These enzymes were not measured during long-term starvation due to their short time of activity after the cells became NC and the ability of the whole-genome arrays to measure the expression of the proteins needed for these processes. Since the AP activity decreased, yet the cells produced end products from amino acid metabolism, we measured amino acid concentration in the medium.

Amino acid metabolism. Amino acid transport is facilitated by antiport, PMF, and ATP-driven mechanisms in lactococci (31). Therefore, amino acid metabolism and transport were determined as a measure of active metabolism, PMF, and ATP availability for transport of small molecules. The amino acids glycine/valine, threonine, tyrosine, alanine, histidine, proline, cysteine, isoleucine, phenylalanine, leucine, aspartate, glutamate, and asparagine did not significantly ($P > 0.05$) change over the incubation time and cellular phase changes.

Arginine decreased significantly ($P \leq 0.05$) to nondetectable levels after 2 days in CDM with ML3 (Fig. 3), as did glutamine over 42 days of incubation (Fig. 3). Conversely, the methionine and serine concentrations significantly ($P \leq 0.05$) increased over time in ML3 (Fig. 3). The reduction of arginine after lactose depletion is consistent with the results of a previous study (8), as is the production of methionine and serine with results of studies of cheese and defined media (32, 57). The cells retained the ability to transport amino acids under all conditions that demonstrate that the PMF (glutamine), ATP availability (serine), and antiporters (arginine) are operable during all time points of starvation. To complement this observation, we determined the ability of the NC cells to metabolize amino acids that will yield end products, energy, redox control, and a PMF gradient.

Amino acid catabolism in NC cells. Amino acids were transported and are a substantial source of ATP during starvation (20, 21). BCAAs are imported via a PMF-dependent transport system, which requires an intact membrane to maintain the potential (31), resulting in the production of 2 mol of ATP/BCAAs. We observed BCAA transport (PMF dependent) and metabolism during this study, further supporting previous observations of intact membranes by using fluorescence. Therefore, NC cells were collected from all strains and assayed for fatty acid production using [2-^{13}C]L-leucine nuclear magnetic resonance after incubation for 3.5, 2, and 2 years, respectively, for ML3, SK11, and IL1403. All strains catabolized leucine to isobutyric, propionic, and acetic acids. Intracellular leucine was found with the concomitant presence of isobutyric acid in the supernatant, indicating that this amino acid was being converted to a new product (i.e., branched-chain fatty acids) that was found only during starvation (20), which is consistent with the observations of Ganesan et al. (21). This confirmed our hypothesis that amino acids were transported via a PMF-dependent mechanism for the generation of ATP during sugar starvation in short- and long-term starvation studies, suggesting that this is an important mechanism for survival without sugar. Taken together, these experiments verify that NC cells have an intact membrane that is capable of supporting substrate transport via three different mechanisms and enable amino acid metabolism for new end products that result in ATP generation to support survival and cellular metabolism.

Gene expression analysis. The biochemical and physiological data provided evidence that the cells were capable of survival and metabolism. However, it was unclear whether this was from enzymes produced during logarithmic-phase growth or from transcription and subsequent translation during starvation; as such, we determined the gene expression at key phenotypic points during the experiment with IL1403 by using a whole-genome expression chip we designed previously (21). This study investigated the genes annotated for glycolysis, cell division, autolysis, and their known regulators. Using a repeated-measure statistical model, we found that 34 of the 300 genes in these categories were significantly ($q \leq 0.3$) regulated at sugar exhaustion (SE) and nonculturability (Table 1). The specific metabolic functions associated with these two cellular stages are further described with respect to their role in cellular function and metabolic ability.

Glycolysis. The genes associated with sugar transport and catabolism were regulated differently between SE and attainment of nonculturability (Table 1). Many of the glycolytic genes involved in catabolizing glucose to triose phosphates were repressed during the incubation time but did not change (i.e., <2-fold change) at SE (Table 1). For example, the *pts* genes associated with the phosphorelay system for phosphotransferase transport system (PTS) sugar transport poise the cell for rapid sugar use and were regulated differently as the cell progressed to the NC state. The kinase (*ptsK*) and Hpr carrier protein (*ptsH*) genes were repressed at NC attainment (Fig. 4). This was somewhat expected, as glucose exhaustion represses the genes associated with sugar metabolism (17), but the progression of repression was not expected. Additional

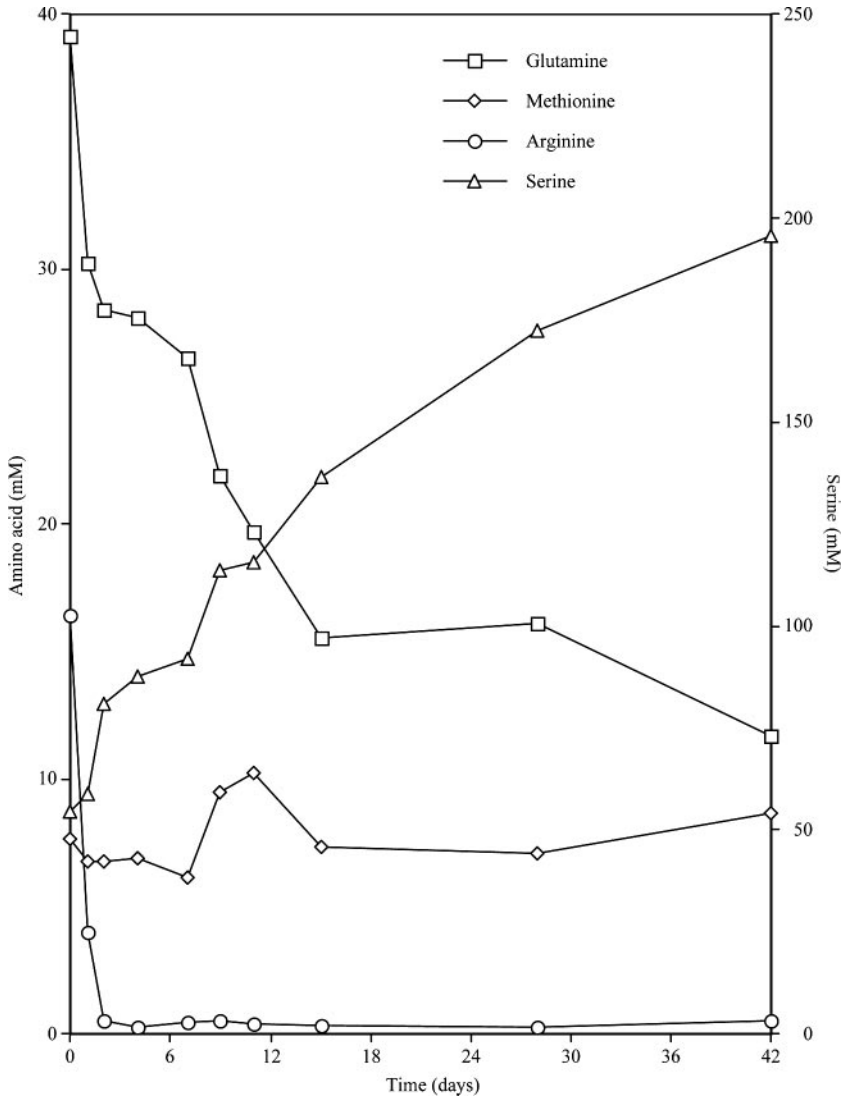

FIG. 3. Extracellular amino acid profile for ML3 grown in CDM with 0.1% lactose. Serine concentrations are depicted on the *yy* axis and all other amino acid concentrations on the *y* axis. The coefficient of variation ranged between 1 and 8% over all time points.

uncharacterized genes related to sugar transport (*yngE*, *ypcG*, and *malFG*) reflect the redundancy (Table 1).

The genes for other steps of glycolysis, for example, *pgk* (phospholycerate kinase) and *yjhF* (phosphoglyceromutase), were significantly ($q \leq 0.3$) induced 2.5- to 4-fold above the median expression level during starvation. Only six genes remained unchanged from logarithmic-phase growth to nonculturability (*ptsI*, *lacR*, *galK*, *pmg*, *pflA*, and *pta*). Notably, the unregulated genes are all associated with a regulatory protein, sugar phosphorylation, or sugar transport via a permease, while starvation repressed some genes needed to utilize ATP for sugar phosphorylation. Additionally, some of the downstream products of glycolysis (3-phosphoglycerate, phosphoenol pyruvate, and pyruvate) are also substrates for interconversion to amino acid biosynthesis, which is consistent with the observations of amino acid production and the subsequent metabolism to branched-chain fatty acids. This suggests that metabolic mechanisms in glycolysis that scavenge precursors for protein synthesis, redox, and ATP generation remain active during starvation and nonculturability from new transcription and translation events. This also indicates that the cells may possess intermediates that contribute to depletion of the PEP potential and, hence, are energetically poised to shuttle glycolytic intermediates that metabolize substrates into amino acids for subsequent energy. These observations are consistent with the observation of peptide metabolism after 3.5 years of carbohydrate starvation and BCAA catabolism for the production of ATP.

ATP synthase. Since glycolytic genes were repressed, ATP synthesis must have occurred from alternate sources. One such source is the use of ATP synthesis as part of macromolecule transport that is required for cellular processes important in survival and metabolism. The F_0F_1 ATP synthase of lactococci acts as a proton pump that maintains intracellular PMF gradient by utilizing energy from ATP hydrolysis to pump protons out of the cell (30). The F_1 unit of ATP synthase (i.e., the ε

TABLE 1. Changes (*n*-fold) of genes related to different functional categories

Function and gene	Description	Fold change ratio[a]	
		T_{SE}/T_0	T_{NC}/T_{SE}
Glycolysis			
ptsH	Phosphocarrier protein Hpr	1.3	**−1.4**
ptsI	PEP protein phosphotransferase (EC 2.7.3.9)	1	1.2
ptsK	Hpr(Ser) kinase (EC 2.7.1.-)	−1.2	**−1.9**
yrcA	Phospho-beta-glucosidase (EC 3.2.1.86)	−1.7	1.8
lacR	Lactose transport regulator	1	1.1
lacZ	Beta-galactosidase (EC 3.2.1.23)	−1.2	1.2
galK	Galactokinase (EC 2.7.1.6)	1.3	1.4
galT	Galactose-1-phosphate uridylyltransferase (EC 2.7.7.10)	1	−1.2
glk	Glucose kinase (EC 2.7.1.2)	1.5	−1.1
ypbG	Sugar kinase (EC 2.7.-.-)	−1	1.6
lacC	Tagatose-6-phosphate kinase (EC 2.7.1.1)	1.6	**−3.1**
galE	UDP-glucose 4-epimerase (EC 5.1.3.2)	1.3	−1.3
galM	Aldose-1-epimerase (EC 5.1.3.3)	**2.4**	−1.8
pfk	6-Phosphofructokinase (EC 2.7.1.11)	−1.3	−1
fbaA	Fructose-bisphosphate aldolase (EC 4.1.2.13)	1	−1.4
pgmB	Beta-phosphoglucomutase (EC 5.4.2.6)	1.7	−1.4
femD	Phosphoglucosamine mutase (EC 5.4.2.2)	1.6	−1.2
tpiA	Triosephosphate isomerase (EC 5.3.1.1)	−1.1	**−2.3**
dhaK	Dihydroxyacetone kinase (EC 2.7.1.2)	1.2	**−3.4**
dhaL	Dihydroxyacetone kinase (EC 2.7.1.2)	−1.2	1.2
dhaM	Dihydroxyacetone kinase (EC 2.7.1.2)	1	−1.1
enoA	Enolase (EC 4.2.1.11)	1.6	−1.1
enoB	2-Phosphoglycerate dehydratase (EC 4.2.1.11)	2	−1.9
pgiA	Glucose-6-phosphate isomerase A (EC 5.3.1.9)	1	**−1.8**
gapA	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	−1.1	1.2
gapB	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	−1	−1.9
pgk	Phosphoglycerate kinase (EC 2.7.2.3)	**1.3**	**−1.2**
pmg	Phosphoglycerate mutase (EC 5.4.2.1)	1.3	1.2
yrjI	Phosphoglycerate mutase (EC 5.4.2.1)	−1.3	1.4
yjhF	Phosphoglycerate mutase (EC5.4.2.1)	**1.7**	**−1.2**
pyk	Pyruvate kinase (EC 2.7.1.40)	1.7	−1.6
ldh	L-Lactate dehydrogenase (EC 1.1.1.27)	−1.6	1.3
ldhB	L-Lactate dehydrogenase (EC 1.1.1.27)	−1.3	1.2
ldhX	L-Lactate dehydrogenase (EC 1.1.1.27)	2.7	−1.2
pdhA	Pyruvate dehydrogenase E1 component alpha subunit (EC 1.2.4.1)	1.3	−1.5
pdhB	Pyruvate dehydrogenase E1 component beta subunit (EC 1.2.4.1)	1.1	−1.3
pdhC	Dihydrolipoamide acetyltransferase component of PDH complex (EC 2.3.1.12)	−1.5	1.6
pdhD	Lipoamide dehydrogenase component of PDH complex (EC 1.8.1.4)	−1.6	1.2
poxL	Pyruvate oxidase (EC 1.2.3.3)	1.1	−1.3
nifJ	Pyruvate-flavodoxin oxidoreductase (EC 1.2.7.-)	1	−1
pfl	Pyruvate-formate lyase (EC 2.3.1.54)	−1.1	1.3
pflA	Pyruvate-formate lyase-activating enzyme (EC 1.97.1.4)	1	1.1
pta	Phosphate acetyltransferase (EC 2.3.1.8)	1.2	1.1
ackA1	Acetate kinase (EC 2.7.2.1)	1	−1.4
ackA2	Acetate kinase (EC 2.7.2.1)	1.6	−1.2
ypcG	Sugar ABC transporter substrate binding protein	**2.2**	**−1.8**
yngE	Sugar ABC transporter ATP binding protein	1.2	1.5
mleP	Malate transporter	1.8	1.1
malF	Maltose ABC transporter permease protein	**−2.5**	**−2.2**
malE	Maltose ABC transporter substrate binding protein	1.5	**2.4**
ccpA	Catabolite control protein A	1	2.0
ATP synthesis			
atpA	ATP synthase α subunit (EC 3.6.1.34)	0	−1.3
atpD	ATP synthase α subunit (EC 3.6.1.34)	0	−1
atpH	ATP synthase δ subunit (EC 3.6.1.34)	0	−1.3
atpE1	ATP synthase ε subunit (EC 3.6.1.34)	**1.9**	**−1.9**
atpE2	ATP synthase ε subunit (EC 3.6.1.34)	0	**1.4**
atpG	ATP synthase γ subunit (EC 3.6.1.34)	0	−1.8
atpB	ATP synthase subunit a (EC 3.6.1.34)	0	−1.1
atpF	ATP synthase subunit b (EC 3.6.1.34)	0	−1.1
pstA	Phosphate ABC transporter ATP binding protein	−1.2	−1
pstB	Phosphate ABC transporter ATP binding protein	**−3.5**	**−2.8**
Protein metabolism			
oppA	Oligopeptide ABC transporter substrate binding protein	**−1**	−1.3

Continued on following page

TABLE 1—*Continued*

Function and gene	Description	Fold change ratio[a]	
		T_{SE}/T_0	T_{NC}/T_{SE}
oppB	Oligopeptide ABC transporter permease protein	1.2	−2.7
oppC	Oligopeptide ABC transporter permease protein	−1.6	−1
oppD	Oligopeptide ABC transporter ATP binding protein		−1.3
oppF	Oligopeptide ABC transporter ATP binding protein	−1.2	−1.1
optA	Oligopeptide ABC transporter substrate binding protein	0	−3
optB	Oligopeptide ABC transporter permease protein	1.5	1.3
optC	Oligopeptide ABC transporter permease protein	**−1.7**	**−1.8**
optD	Oligopeptide ABC transporter ATP binding protein	0	−1.8
optF	Oligopeptide ABC transporter ATP binding protein	0	−4.2
optS	Oligopeptide ABC transporter substrate binding protein	1.1	1.6
ydcB	Amino acid ABC transporter ATP binding protein	1.2	1
yjgE	Amino acid ABC transporter ATP binding protein	−1.3	1.3
yjgD	Amino acid ABC transporter permease protein	−1.1	1.2
yjgC	Amino acid ABC transporter substrate binding protein	−3	−1.6
yvdF	Amino acid ABC transporter substrate binding protein	2.2	−1.3
ydcC	Amino acid ABC transporter permease protein	1	−1
yrfD	Amino acid antiporter	−1.3	−1.4
yagE	Amino acid permease	1.1	**2.8**
ydgB	Amino acid permease	2.5	−1.1
ydgC	Amino acid permease	−1.1	1.6
yibG	Amino acid permease	**−1.6**	**−1.8**
ylcA	Amino acid permease	**2.5**	−1.2
yqfD	Amino acid permease	−1	−1.4
yshA	Amino acid permease	−1.1	1.1
ysjA	Amino acid permease	−1.1	−1
dtpT	Di-/tripeptide transporter	**−2.5**	1.5
pepA	Glutamyl aminopeptidase	1.7	1.2
pepC	AP C	0	2
pepN	AP N	0	1.1
pepP	AP P	0	−2.5
pepM	Methionine AP	0	1
pepXP	X-prolyl-dipeptidyl AP (EC 3.4.14.5)	0	1.3
pepDA	Dipeptidase	0	1
pepDB	Dipeptidase	0	2.2
pepQ	Proline dipeptidase (EC 3.4.13.9)	0	−1
pepV	Dipeptidase	2.3	−1
pepF	Oligoendopeptidase F	0	−1.7
pepO	Neutral endopeptidase	0	1
pepT	Tripeptidase	0	−1.7
Cell lysis and division			
acmA	*N*-Acetylmuramidase (EC 3.5.1.28)	1.4	1.8
acmB	*N*-Acetylmuramidase (EC 3.5.1.28)	1.1	−1.2
acmC	*N*-Acetylmuramidase (EC 3.5.1.28)	−1.6	−1.1
acmD	*N*-Acetylmuramidase (EC 3.5.1.28)	2	−1.3
ftsX	Cell division protein	1	**−1.2**
ftsA	Cell division protein	−1.7	**−1.2**
ftsH	Cell division protein	−1.1	**−1.3**
ftsK	Cell division protein	1.3	**−2.7**
ftsQ	Cell division protein	1.1	1.3
ftsW1	Cell division protein	−1	−1.4
ftsW2	Cell division protein	−2.5	1.3
ftsY	Cell division protein	**−2.1**	−1.1
ftsZ	Cell division protein	−1.2	**−1.3**
ftsE	Cell division ATP binding protein	1	−1.1
gidA	Glucose-inhibited division protein		1.4
gidC	Glucose-inhibited division protein		1.3
gidB	Glucose-inhibited division protein		1.3
murA1	UDP-*N*-acetylglucosamine-1-carboxyvinyltransferase	−1.5	1.2
murA2	UDP-*N*-acetylglucosamine-1-carboxyvinyltransferase	**−4**	1.1
murB	UDP-*N*-acetylenolpyruvoylglucosamine reductase	1	−1.3
murC	UDP-*N*-acetylmuramate-alanine ligase (EC 6.3.2.8)	**−1.8**	**2.4**
murD	UDP-*N*-acetylmuramoylalanine-d-glutamate ligase (EC 6.3.2.9)	**2.8**	−1.2
murE	UDP-murnac-tripeptide synthetase	−1.3	−1.1
murF	D-Ala-D-Ala-adding enzyme (EC 6.3.2.15)	−1.2	**−1.9**
murG	Peptidoglycan synthesis protein MurG	−1.3	−1

Continued on following page

TABLE 1—*Continued*

Function and gene	Description	Fold change ratio[a]	
		T_{SE}/T_0	T_{NC}/T_{SE}
dnaA	Replication initiation protein DnaA		−1
dnaB	Replication protein DnaB		1
dnaC	Replicative DNA helicase		**−2.1**
dnaD	DNA replication protein DnaD		−1.1
dnaE	DNA polymerase III, alpha chain 2 (EC 2.7.7.7)		**−2.3**
dnaG	DNA primase (EC 2.7.7.-)		−1.1
dnaH	DNA polymerase III, subunits beta and tau (EC 2.7.7.7)		1.1
dnaI	Primosomal protein DnaI		**3.7**
dnaN	DNA polymerase III, beta chain (EC 2.7.7.7)		−1
dnaQ	DNA polymerase III, epsilon chain		1.1

[a] The changes (*n*-fold) of significantly changing genes are in boldface. All significantly changing genes have a q value of ≤0.3. Positive values of change indicate induction of gene expression, while negative values indicate repression. T_0, initial time point; T_{SE}, time point of SE; T_{NC}, time point of nonculturability.

subunit *atpE1*) was significantly induced ($q \leq 0.3$) at SE, while gene expression was low and remained unchanged for the α, β, γ, and δ subunits. The genes for the F_0 unit responsible for proton channel activity (*atpB* and *atpF*) were expressed during glucose availability and SE. The α, β, and γ subunits are responsible for ATP hydrolysis for proton translocation while the ε subunit is responsible for connecting channel activity to proton translocation (45). The low expression of the α, β, and γ subunits suggests that the cells are reducing ATP hydrolysis during starvation to minimize ATP loss by proton translocation yet expressing these proteins at a sufficient level to maintain the PMF, as indicated by amino acid transport.

Protein metabolism. Protein metabolism, which includes peptide and amino acid transport, proteases, and APs, provides amino acid substrates for metabolic processes. The most notable observation is the compete repression of *opp*, which is the primary peptide transport system used during log-phase growth (Table 1). This strain has a portion of the *opp* system on

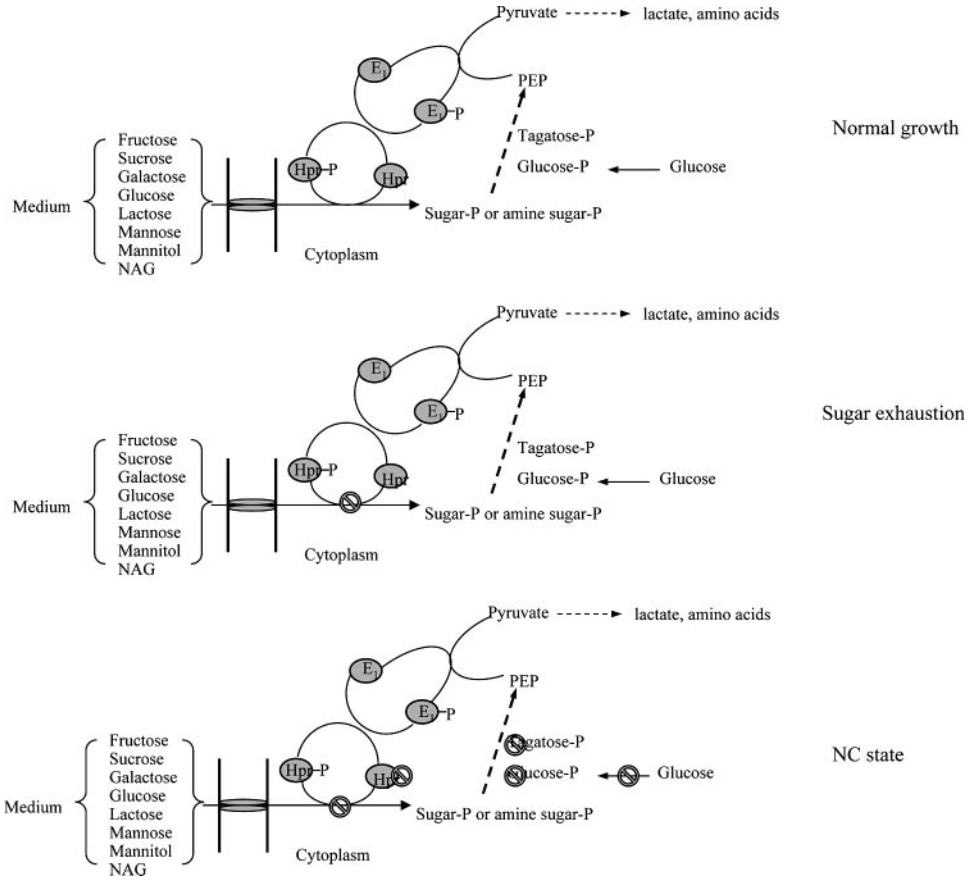

FIG. 4. Schematic representation of gene expression of the lactococcal PEP-dependent PTS for various PTS sugars with connections to glycolytic intermediates. The symbol (⊘) indicates gene repression. NAG, *N*-acetylglucosamine.

the chromosome that is inactive due to mutations in the regulator sequences upstream of the structural genes (60). An alternative peptide transport system (*opt*) was differentially regulated during SE and the NC state that allowed small peptides to be transported during nonculturability in place of *opp*. Among all peptidase and peptide transport genes, *optC* was the only gene significantly repressed ($q \leq 0.3$) at sugar depletion and nonculturability. The oligopeptide transporter genes *optB* and *optS* were expressed across sugar availability, sugar depletion, and attainment of nonculturability. The *dpt* genes were also expressed under both pH conditions. Therefore, the genes needed for short-chain substrate transport, specifically oligopeptides and amino acid transport systems, were transcribed during sugar starvation and nonculturability, as observed in the biochemical studies (Table 1). Additionally, the induction of *codY*, the transcriptional regulator repressed by high BCAA pools (27), also verifies that these transporters were involved in amino acid transport and that active gene regulation was occurring during starvation and nonculturability.

Among the AP genes, the expression of *pepXP* (X-prolyl-dipeptidyl AP) was high in both glucose availability and starvation. Notably, *pepN*, the gene for the enzyme used as the hallmark of lactococcal lysis, was not expressed in any phase. The enzyme activities of APs are typically associated with cellular lysis of lactococci in the cheese matrix (6, 11, 12). The enzyme activities of APs in this study declined to nondetectable limits during starvation. This was caused by repression of these genes when lactose was depleted. This is verified by the high level of expression of the pleiotropic regulator *codY*, which, when expressed, senses the availability of pools of BCAAs and represses AP gene expression (27).

Cell division. The *fts* genes code for proteins that form the cytoskeleton and are required for cell division bacteria. In *L. lactis* IL1403, while some genes are properly annotated, at least five genes (*pbpX*, *yacC*, *yiiI*, *yraB*, and *ytdB*) that were originally not assigned to cell division were assigned in the SK11 genome (35). Analysis of the protein domains within the ERGO bioinformatics suite identified the proteins of these genes to be homologues of the cell division proteins or regulators (FtsI, DivIVC, FtsL, DivIVB, and DivIVA, respectively) in IL1403. This indicates that *L. lactis* IL1403 contains the full complement of cell division genes and not just a partial list as initially annotated.

Gene expression analysis of the cell division genes showed that many of these genes were either repressed or not induced during starvation. Although a partial set of genes was induced in the NC state, *ftsZ*, which is involved in the formation of a tubulin structure for cell division, was repressed. The only other gene that has a known function is *ezrA*, which is involved in ring formation during the septation process. This gene was significantly induced ($q \leq 0.3$) >5-fold at the onset of nonculturability. While an exact scheme of the role of each individual gene is not identified in lactococci, the critical role of each gene during the cell division process is widely accepted. The repression of many of these genes suggests that cell division, as a process, is not functional under sugar exhaustion or NC conditions.

Cell lysis. Cell wall remodeling and repair are facilitated by the *acm* gene family, which also plays a role in lysis in lactococci (5, 28, 47). Additionally, lactococci contain bacteriophage and prophage elements that are activated during stress (2) that also cause lysis. As such, the genes associated with these events are thought to be induced during stationary phase and cellular stress and lead to lysis (5, 27, 46). The *N*-acetyl muramidases (*acmABCD*), which are directly responsible for autolysis in lactococci, were not expressed at any phase in this study (Table 1) (5, 28, 47). Additionally, during the same cellular phase in glucose starvation, *acmB* was significantly repressed ($q \leq 0.3$) 2.5- to 4-fold. Interestingly, holins from three different prophage families (pi148, pi251, and pi306) were not expressed during any of the growth states in this study. Further, the cell wall biosynthetic *mur* operon was not expressed during the NC state. Taken as a whole, these data indicate that cell division and cell wall repair slowed or stopped and that the autolytic genes used to lyse the cells were not induced. These observations support the observations that substantial portions of the cells were intact during the 3-year incubation so that the cells stopped dividing without lysing or dying.

DISCUSSION

The NC state in bacteria is a complex and controversial topic. No direct approaches to identify live, metabolically active bacteria that cannot form colonies on a plate are available to directly prove the existence of this state. Consequently, multiple lines of diverse evidence are needed to confirm the existence of this cellular state, as opposed to colony formation, cell death, or lysis in combination with metabolism. The lines of evidence provided in this study demonstrated that sugar starvation induced lactococci to become NC with metabolic activity for at least 3 years. Stuart et al. (48) found indications of this ability during short-term starvation (i.e., 14 days), with the same production and consumption of amino acids from the medium. This study extends those observations to multiple years and describes the molecular basis for this cellular state.

Each strain tested entered the NC state during the study. The exact times of entry into the NC state for the strains were different and significantly ($P \leq 0.05$) influenced by time, carbohydrate, and medium pH. This was also observed by Stuart et al. (48) with a strain that slowly depleted the lactose from the medium. Decreasing the medium pH significantly ($P \leq 0.05$) shortened the time taken for the metabolism of lactose and the onset of nonculturability in SK11, but it did not impact the ability of ML3 to become NC and remain metabolically active during long-term starvation (Fig. 1). Interestingly, reduction of the pH to 5.2 with IL1403 significantly ($P \leq 0.05$) delayed the onset of nonculturability (Fig. 1C). The pH responses of strains differed by their sugar utilization, indicating that the ability to use carbohydrates is the primary factor in the response to starvation and the impact on survival. These observations point to differences in the regulation of sugar metabolism, including transport via PTS and permease transporters. Varying the pH modified only the time to enter nonculturability but not the long-term biochemical capabilities during nonculturability. This has implications for the commercial application of these organisms. For example, during cheese ripening, the pH is usually 4.8 to 5.2. As such, the metabolic changes associated with the NC state will vary as a function of the pH of the matrix; hence, the flavor dynamic will also vary in unpredictable ways.

The production of ATP in the cells was observed during all cellular phases in this study. This finding is important since ATP-dependent transport of nutrients and metabolic processes allow the cell to produce new compounds or secondary metabolites. Ganesan et al. (21) noted the parallel between glycolysis and BCAA utilization with respect to ATP production, allowing the pathways to be divided into two halves. The initial half (i.e., the preparatory phase) of glycolysis provides intermediates that switch between sugar and amino acid use during starvation to provide energy (i.e., the payoff phase). Glucose metabolism genes of the preparatory phase were repressed during starvation and nonculturability, presumably due to the exhaustion of glucose (Fig. 4). The genes of the late phase are involved in reactions that produce substrates for amino acid metabolism. These genes were expressed at starvation and nonculturability. Sugar metabolism is repressed by CcpA, which activates the metabolism of nitrogenous substrates in *Firmicutes* (7). Since lactococci lack functional sigma factors to regulate metabolism, the exact cadre of regulatory events is unclear. However, the involvement of CcpA and its cognate binding site (CRE) in the metabolic shift is clear. Interestingly, all of these strains contain multiple CRE sites upstream of selected genes of the arginine deiminase pathway, which are likely activated differentially by CcpA. Chou (8) found that the proteins of the arginine deiminase pathway were produced differently, but not in an operon, and resulted in the metabolism of Arg in a segmented fashion. CcpA mediates catabolite control by binding to HPr-SerP (7, 37), which in turn needs to be phosphorylated by PEP. Consequently, depletion of the HPr-SerP intermediate is likely since the phosphocarrier protein (*ptsH*) and the Hpr(Ser) kinase (*ptsK*) were repressed, leaving CcpA available to bind other regulatory partners that are unrelated to sugar metabolism.

During the transition from growth to the NC state, the cell repressed division genes, remained intact during environmental stress, and remained metabolically active to produce secondary metabolites. The majority of the *fts* genes (8 or 10) were repressed during this transition (Table 1), thereby leaving the cell unable to initiate cell division via *ftsZ*. Coupled with that was the repression of the genes associated with DNA replication (Table 1); consequently, the cells lost the ability to divide. Interestingly, the genes for cell division and DNA replication also contained upstream CRE sites, indicating that these genes were regulated by CcpA. This study found an inverse correlation in gene expression between these genes: *ccpA* was induced as *fts* (specifically *ftsZ*) and *dnaN* were repressed.

The cellular integrity was maintained via intact membranes and the lack of induction of the lytic system (*acm* or phage holins) (Table 1). While none of the autolytic genes contain upstream CRE sites, many of them were repressed while *ccpA* was induced, indicating that additional regulatory systems are involved in autolysis. While other putative sugar regulators had different expression patterns, only the role of CcpA in glycolytic repression was confirmed. Further studies are needed to verify the exact role of the individual regulators in this multigene change.

With the demonstration of NC cells, the repression of PTS sugar transport, and the shift to nitrogen substrates, the extent of metabolic ability remained. The proteolytic system was regulated in mixed directions during the incubation. Oligopeptide transporter (Table 1) expression was differentially regulated as the cell entered starvation and nonculturability. Some components were repressed while others were induced. Three peptide transport systems exist in lactococci, *opp*, *dpt*, and *opt* (10). Only the regulation and action of *opp* are well characterized (18). In this study, *opp* was repressed while *dpt* and *optBCS* were concomitantly induced upon entry into starvation and nonculturability. This study demonstrated that $\alpha s_{1\text{-}9}$ casein was transported during nonculturability, presumably via *opt*. Interestingly, the induction of *dtp* was associated with the induction of three dipeptidases (*pepXP*, *pepDA*, and *pepDB*), while two other dipeptidases (*pepQ* and *pepV*) were repressed. The induction of these functionally redundant systems allows the cell to acquire protein-based substrates with the repression of PTS sugar transport to provide energy to the cell, but additional studies are needed to functionally characterize their regulation and role in NC physiology.

The transport of amino acids regulates APs and peptide metabolism via the pleiotropic repressor CodY (40). During this study, only *pepCDTO* was induced during nonculturability; the remaining APs were repressed. This observation verifies the activity of APs increasing during growth but declining over starvation (Table 1). This was supported by the reduction in enzymatic activity for these proteins. Two uncharacterized amino acid transporters were induced during nonculturability, while many others were repressed. Additional studies of these uncharacterized transporters are needed to determine their role in amino acid utilization, regulation, and substrate specificity.

However, the linkage between the repression of sugar metabolism and the induction of amino acid utilization is demonstrated in this study. The data from this study and the results of Chou (8), where ML3 metabolized arginine only after the cell depleted lactose, are in agreement. This switch was regulated by the repression of sugar utilization via CcpA and was induced before lactococci shifted to amino acid metabolism from sugar metabolism (33). These similar responses suggest that there is a minimal set of metabolic abilities that allow the cell to survive long-term carbohydrate starvation that are linked to the shift of metabolism from sugar to amino acids, which provide energy in the absence of electron transport capabilities in these organisms. All the strains maintained constant levels of ATP for long periods of carbohydrate starvation, even after becoming NC within 7 days of carbohydrate depletion. This indicates that the cells have enough energy to conduct central metabolic reactions that require energy, such as enzyme activity and transport, which was verified by measuring gene expression and by phenotypic determination of amino acid and peptide metabolisms. The intermediates of these reactions are renewed by the interconnection between glycolytic intermediates and amino acid metabolism. Interestingly, the cell seems to conserve energy by repressing ATP synthase subunits responsible for ATP hydrolysis (Table 1) associated with proton pumps, which channel ATP into intracellular processes.

During the 42-day starvation, the cultures became NC on solid agar after the ATP concentration dropped below 0.5 pM (Fig. 1 and 3). We presumed that this represented the maintenance energy required for lactococci to remain culturable on solid agar, as did Stuart et al. (48). However, this supposition

was not true during long-term starvation, where ATP consistently remained above 100 pM throughout the NC period. The difference in the minimal ATP during long-term starvation may be associated with the presence of BCAAs at 10-times-higher levels than for short-term starvation. The presence of additional BCAAs during the long-term starvation resulted in 1,000- to 10,000-fold-higher levels of ATP (Fig. 2) than for cells without BCAA, thus confirming the shift from sugar to amino acid metabolism to provide ATP, even though the NC state was attained within the same time interval (Fig. 1). This change was made for long-term starvation experiments to test whether these amino acids were able to increase ATP levels. The positive result led us to conclude that BCAAs may support the carbon and energetic needs of the cells during long-term starvation at levels that were similar to those during lactose utilization.

Logarithmic-phase cells of lactococci utilize BCAAs to produce straight and branched-chain fatty acids in the absence of sugar (22). These pathways also allow for the production of ATP by substrate-level phosphorylation and generation of NAD that can subsequently be used in redox-mediated biosynthetic pathways for additional ATP (22, 23). *Brevibacterium linens* utilizes BCAAs only after carbohydrate starvation (23). These observations provide evidence that BCAAs extend survival via additional ATP and support precursor generation for other pathways to be functional during starvation. Arginine was depleted before the onset of the NC state (Fig. 3). Under such energy-depleted conditions, BCAAs may be the substrates that support energy, carbon, and nitrogen requirements in long-term starvation and regulate the expression of *codY* (40) to control the peptidase system (27).

The concomitant depletion of arginine and lactose is in agreement with the results of previous studies done both under starvation conditions and with ripening cheese, in which the arginine concentration decreased (41, 49, 57). Arginine is used for growth requirements (31), the maintenance of pH homeostasis (8), and the maintenance of ATP. The transport of glutamine is energy driven via phosphate, presumably through the use of ATP or other energy-rich phosphate intermediates (31). The consistent decrease of glutamine over time under all conditions (Fig. 3) indicates that alternate energy sources are being utilized to generate ATP, which is used in transport.

Methionine is produced during cheese ripening (32, 57) as well as at the onset of starvation (Fig. 3). This amino acid is associated with desirable sulfur notes in cheddar cheese and is linked to the production of volatile sulfur compounds (59). It is not known why methionine increases during carbohydrate starvation, but it may be linked to nucleic acid metabolism via *S*-D-ribosyl-L-homocysteine or *S*-adenosyl-L-methionine produced by lactococci (2). Serine, the precursor to methionine, is also released during the ripening of cheese (20, 32, 57), starvation (19), and nonculturability (Fig. 3). These observations suggest that mechanisms to degrade serine may not be active during starvation, as lactococci contain genes for the enzymes of serine catabolism. Hence, methionine and serine production may be the biomarker of starvation or nonculturability in lactococci.

The presence of viable cells for up to 3 years, as measured by spectrofluorometry, in combination with low expression of lytic and cell wall repair genes, denotes the maintenance of a cellular membrane that did not undergo lysis and aided the preservation of nucleic acids. This is contrary to the current dogma that states that lactococci die, lyse, and lose viability due to harsh environments (19, 34, 52). The estimation of culturable cells relies on the ability of cells to replicate on solid media. The absence of any culturable counts simply indicates the inability of cells to replicate and not necessarily their death, as demonstrated in this study in combination with transcription and translation events identified with gene expression analysis.

The strong correlations in *ccpA* induction and repression of some glycolytic genes, cell division and autolysis genes, and the induction of amino acid metabolism, along with attainment of the NC state and the maintenance of membrane intactness and ATP levels, suggest that the control of sugar metabolism may be the key to attainment of the NC state in lactococci. The control may exist via mechanisms analogous to catabolite repression/activation mediated by CcpA or other sugar regulators or via mechanisms yet to be identified.

The mechanisms involved in recycling the intermediates between sugar and amino acid metabolisms remain elusive. These cycles provide energy and oxidation/reduction potential for metabolism, yet it is unclear what advantage exporting amino acids to the medium that may be lost due to diffusion provides for metabolism by other organisms in mixed cultures. These data provide a solid foundation to prove that nonculturability exists in lactococci, but the specific details about the regulatory mechanisms between sugar and amino acid metabolisms require further elucidation. As this occurred in all three strains with different plasmid populations and, in one case, a plasmid-free strain (IL1403), it seems likely that the regulatory elements required for nonculturability are encoded on the chromosome.

Conclusions. After carbohydrate depletion, all of the lactococci became NC. Once the cells became NC, they remained intact and lost the ability to use PTS sugar transport but retained the ability to transport protein substrates via ATP- and PMF-dependent mechanisms. This was coupled with a concomitant repression of the genes associated with cellular cytoskeleton, autolysis, or phage-induced lysis. The induction of genes associated with amino acid metabolism led to the production of serine and methionine and the depletion of glutamine, arginine, and leucine. The addition of BCAAs led to the production of new metabolites not found during logarithmic-phase growth. The induction of CcpA correlated with the repression of genes that contained a CRE site upstream of genes related to sugar metabolism, cell division, and cell lysis, and the induction of genes related to arginine catabolism. Taken together, these data prove that lactococci become NC by repressing the production of the cytoskeleton, repressing PTS transport systems, and inducing the metabolism of amino acids that result in ATP and new metabolic products that may be biomarkers of this cellular state.

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